Single Molecule Studies of Eukaryotic Replisomes in Xenopus Egg Extracts

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fluorescence assays and include the fingers-closing transition that has been characterized in structural studies. Using DNA polymerase I (Klenow fragment) labeled with both donor and acceptor fluorophores, we have employed single-molecule fluorescence resonance energy transfer (smFRET) to study the polymerase conformational transitions that precede nucleotide addition. Our experiments clearly distinguish the open and closed conformations that predominate in Pol-DNA and Pol-DNA-dNTP complexes, respectively; minor conformations (corresponding to the closed conformation in the Pol-DNA complex, and the open conformation in the Pol-DNA-dNTP) are also present. By contrast, the unliganded polymerase shows a broad distribution of FRET values, indicating a high degree of conformational flexibility in the protein in the absence of its substrates; such flexibility was not anticipated on the basis of the available crystallographic structures. Real-time observation of conformational dynamics showed that most of the unliganded polymerase molecules sample the open and closed conformations in the millisecond timescale. Ternary complexes formed in the presence of mismatched dNTPs or complementary ribonucleotides show novel FRET species, which we suggest are relevant to kinetic checkpoints that discriminate against these incorrect substrates. Our results advance the mechanistic understanding of the process of nucleotide addition by DNA polymerases and suggest ways to study conformational dynamics in other nucleic-acid polymerases.

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Single Molecule Studies of Eukaryotic Replisomes in Xenopus Egg Extracts

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In eukaryotes, two MCM2-7 helicases are assembled at each origin of replication in the G1 phase of the cell cycle. In S phase, the helicases are activated, leading to assembly of two sister replisomes that replicate DNA in opposite directions. At present, little is known about the spatial arrangement or molecular mechanism of MCM2-7 complexes that are engaged in DNA replication. One scenario is that the two sister MCM2-7 complexes dissociate during initiation and then travel away from one another. Alternatively, the sister helicases might remain physically coupled. To differentiate between such models, we have established a series of single-molecule visualization tools using a nucleus-free replication system of *Xenopus* egg extracts. We demonstrate that these extracts can replicate lambda phage DNA that is mechanically well-stretched and specifically tethered at both ends to a functionalized surface. Our observation of large replication bubbles from single origins on such doubly-tethered DNA argues that helicases located at sister forks can function independently during replication.

In addition, we aim to observe real-time dynamics of different replisome components on doubly-tethered DNAs. For this purpose, we have generated fluorescently tagged Cdc45, an MCM2-7 co-factor that travels with the MCM2-7 helicase. Since the high concentration of labeled Cdc45 needed to support replication causes a high fluorescence background, we tagged Cdc45 with the photoswitchable fluorescent protein mKikGR. The ability to switch on the fluorescence of only those mKikGR proteins that are bound to DNA via Cdc45 enables single-molecule imaging of active replisomes, even at high ambient concentrations of Cdc45-mKikGR. We present the results of initial experiments that prove the feasibility of these techniques as novel ways to study the activity of replication factors in a physiologically relevant environment.

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DNA Base Flipping: New-Found Insights into the DNA Mismatch Recognition Process in E. Coli Muts

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Detection of various base-base mismatches and small insertion/deletion loops in DNA is performed by the MutS mismatch recognition protein. This highly conserved process exists in both prokaryotes and eukaryotes and failure to recognize DNA damage can have a detrimental effect on the fidelity of the genome and, in humans, has been linked to numerous forms of cancer. Several crystal structures have emerged over the years capturing MutS bound to various mismatches. However, the events directly following mismatch recognition remain unclear. To shed light on this matter, we present nine sub-μs, all-atom molecular dynamics simulations of Escherichia coli MutS bound to a G•T mismatch with different nucleotide configurations. From these simulations, we identified significant instability in the adjacent base 5′ to the thymine mismatch. In one case, the 5′ adjacent base completely loses base stacking and flips out spontaneously via the minor groove. To the best of our knowledge, this rare event is the first ever documented case of DNA base flipping from any unrestrained protein-DNA simulation. In addition, these observations are in excellent agreement with a recent experimental study where it was suggested that the 5′ adjacent base could exist in an extrahelical state. To further understand the energetics of base flipping in MutS, we utilized the Hamiltonian replica-exchange molecular dynamics (HREMD) method simulating 44 independent replicas in an explicit water box. The free energy profile generated from HREMD shows two distinct minima, one for the stacked state and one for flipped out state, separated by a small energy barrier with the flipped out state being the more favorable of the two. Together, our results offer an unprecedented level of new insight into the mismatch recognition process and further our knowledge of this complex system.

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DNA Conformational Dynamics in Mismatch Recognition

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DNA mismatch recognition is done by the homodimer MutS in prokaryotes and by its homologues: heterodimers Msh2-Msh3 and Msh2-Msh6 in eukaryotes. Msh2-Msh6 binds preferentially to single insertion/deletions. Msh2-Msh3 has been shown to bind to DNA hairpins. It has been suggested that the conformational dynamics of the DNA substrate (bending and unbending) plays a fundamental role in the recognition process. Mismatch recognition allows identifying a single mismatched DNA pair among thousands of matched basepairs. The process is ATP dependent and different models for DNA discrimination have been proposed based in biochemical evidence as well as AFM studies. In this work we study the conformational dynamics of several DNA substrates and its complexes with the human MutS homologs. The DNA substrates were labeled with fluorescent dyes that constitute a fluorescence resonant energy transfer (FRET) pair. Experiments at the single molecule level allow us to follow the conformational dynamics of the substrates by determining the substrate's end to end distance. We were able to determine the binding and dissociation rates of the proteins from the substrates as well as the conformational state of the substrates under different conditions, including studies with ATP and ADP under both hydrolytic and non-hydrolytic conditions. In particular we discuss the role of the substrate's intrinsic dynamics for binding of hMsh2-hMsh3 to DNA hairpins and DNA 3-way junctions.

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The Dance of Chromosomes during DNA Repair

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DNA repair is an essential process for preserving genome integrity. Among the various forms of DNA damage, double-strand breaks (DSBs) are the most cytotoxic and genotoxic. To repair them, eukaryotic organisms use homologous recombination (HR): it consists of exchanging DNA strands between the broken DNA and an intact homologous DNA and it is choreographed by multi-protein complexes (1). During HR, the search for an intact homologous sequence among the whole genome is the most enigmatic stage (2). How can two homologous needles find each other in the genomic haystack? Is search the result of diffusion and chance encounters, or is there a search apparatus dedicated to bringing the homologous sequences together?

To explore the choreography of the DNA and the recombination proteins during homology search, we developed an *in vivo* 3-colors assay in diploid yeast cells where 2 homologous chromosomes are fluorescently marked at the same locus (with GFP-Lac and RFP-Tet arrays), as well as recombination factors (CFP-tagged proteins). Using deconvolution microscopy, we tracked the movement of the two chromosomes in 3-dimensions in the absence and in the presence of a unique DSB induced near one of the marked chromosome. In the absence of DSB, we found that homologous chromosomes undergo a constrain Brownian motion with a diffusion coefficient of 4.10^{-4} $\mu m^2/s$ inside a small region of 300 nm. When a DSB is induced, the two homologous DNA become highly dynamic and homologous pairing occurs within one hour. This work is the first attempt to visualize simultaneously the movement of two homologous sequences *in vivo* into and out of repair centres.

1. Lisby, M., Barlow, J.H., Burgess, R.C. and Rothstein R. *Cell*: 118, 699-713, 2004.
2. Barzel, A. and Kupiec, M. *Nature*: 9, 27-37, 2007.

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Single-Molecule Measurements of Synthesis by DNA Polymerase with Base-Pair Resolution

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